



**Rapid Fungal Identification by using Matrix-Assisted Laser  
Desorption Ionization-Time of Flight (MALDI-TOF) Mass  
Spectrometry: Focus on Discrimination of  
*Cryptococcus neoformans* and *C. gattii***

Presented by

**Marut Tangwattanachuleeporn**

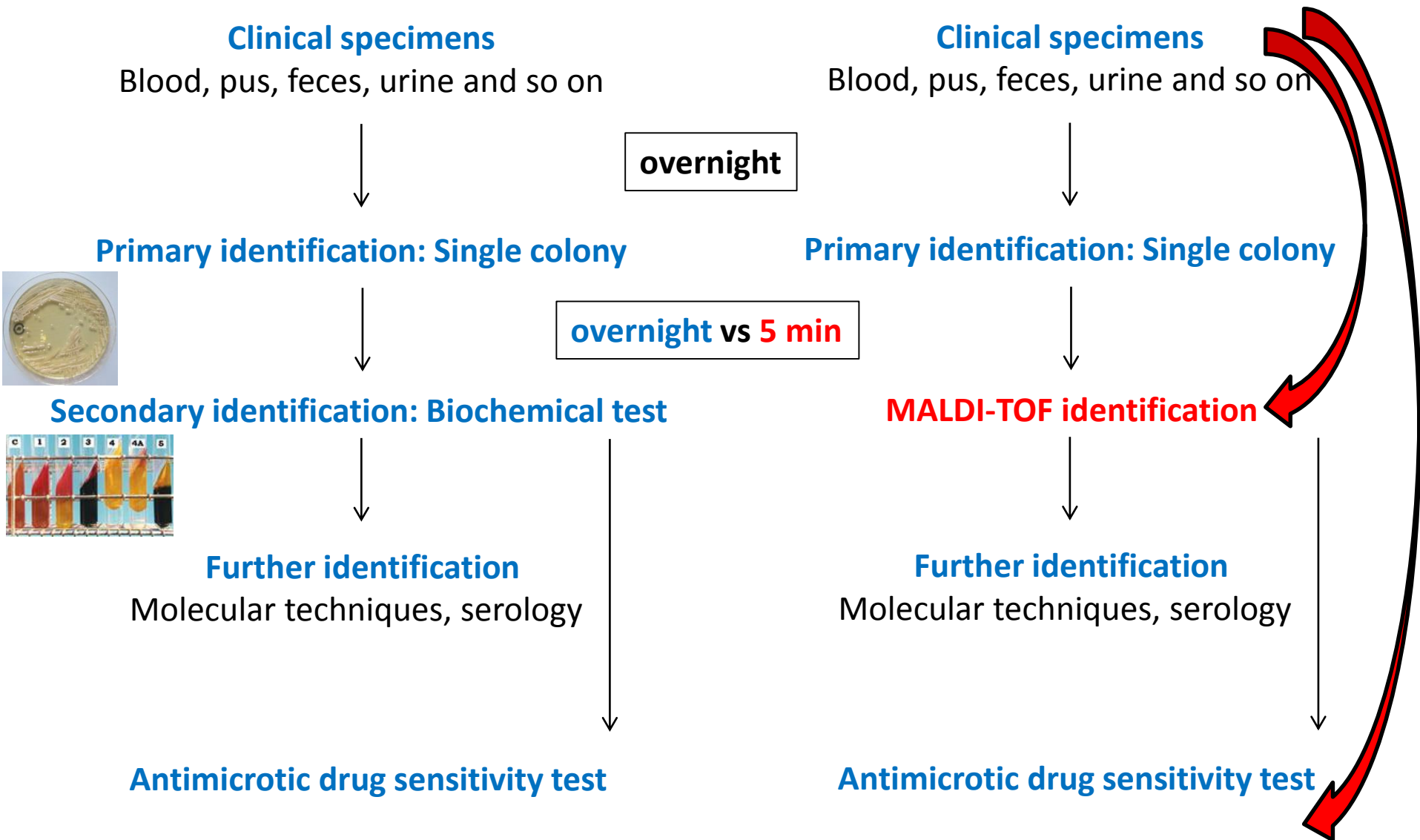
# Outline

- What's MALDI-TOF?
- What's Cryptococcosis and Cryptococcus?
- CryptoType project

Thai-German S&T Cooperation  
Researcher Mobility Scheme



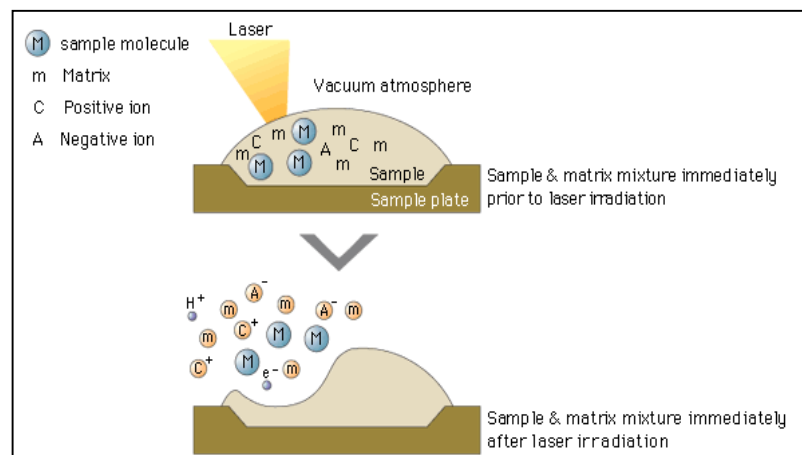
# Process for pathogen identification



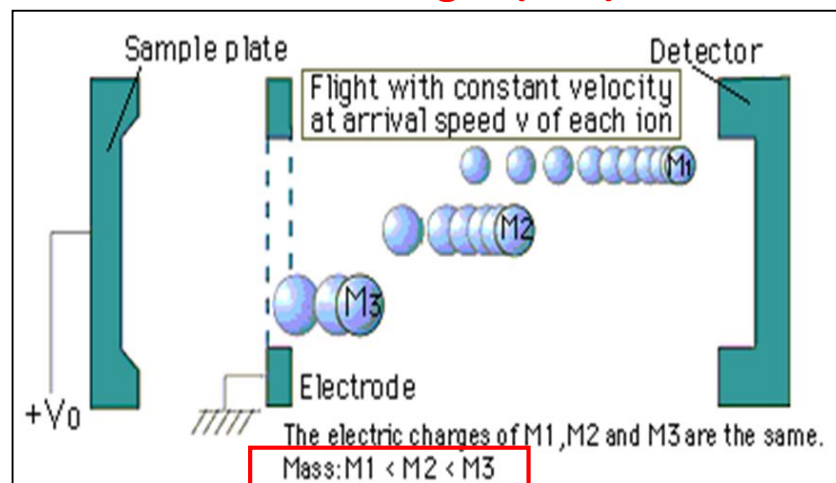
# Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS)



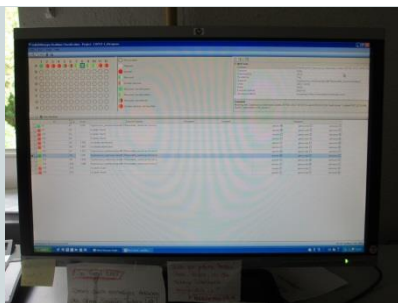
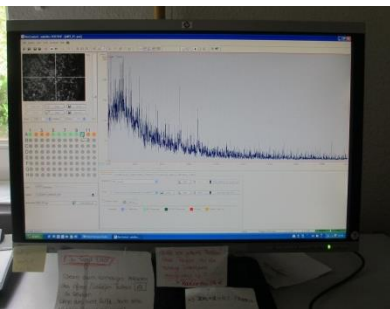
## Matrix Assisted Laser Desorption/Ionization (MALDI)



## Time of Flight (TOF)



# MALDI-TOF MS microorganism identification



**Identified species**

**Data interpretation**

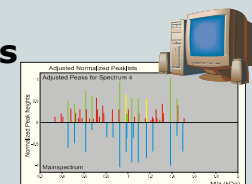
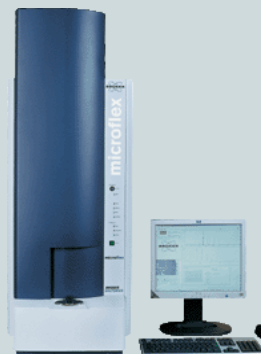
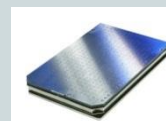
**Generate MALDI-TOF  
profile spectrum**

**Prepare onto  
a MALDI target plate**

**Select a colony**

**Unknown  
microorganism**

?



| Advantages                                             | Limitations                                                          |
|--------------------------------------------------------|----------------------------------------------------------------------|
| • Rapid turnaround time                                | • Requires isolated colony for analysis                              |
| • Automated high throughput (24 or 96 samples per run) | • Lack of susceptibility information                                 |
| • User-expandable database                             | • Currently not FDA approved                                         |
| • Reusable, environment-friendly steel target plates   | • Age of culture may affect identification of some isolates          |
| • Small instrument footprint                           | • Instrument downtime if single instrument                           |
| • Broad applicability to bacteria and fungi            | • Identifications limited by reference spectra in database           |
| • Cost effective (~\$0.50/sample) <sup>3</sup>         | • Inability to differentiate among certain closely related organisms |
|                                                        | • Repeat analysis may be required                                    |

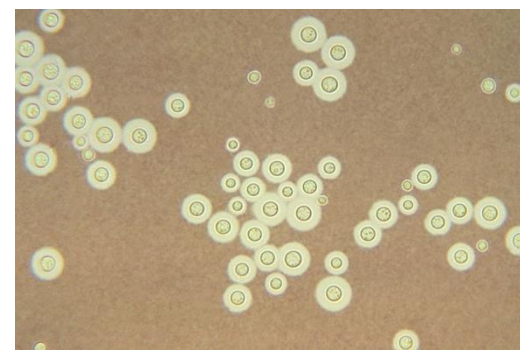
**Table 4.** *Advantages and Limitations of MALDI-TOF Mass Spectrometry Technology*

Taken from Elitza S. Theel in *Communiqué*, Jan 2013

# Cryptococcus



Multiple umbilicated papules and nodules involving the face  
(Mosam A. and Mankahla A., 2009)



*C. neoformans* is an encapsulated yeast

- It is a genus of fungus in group of basidiomycota.
- Its telomorph is *Filobasidiella*
- There are more 30 species but few species cause in human disease. (*C. neoformans*, *C. gattii* and *C. albidus*)
- It causes Cryptococcosis, especially meningitis in AIDS.
- > 50% of Cryptococcosis come from AIDS.

# Opportunistic infection in AIDS patients in Thailand

1. Tuberculosis
2. Pneumocystis carinii pneumonia (PCP)
3. Cryptococcosis
4. Candidiasis
5. Bacterial pneumonia recurrent

Guideline for AIDS treatment in Thailand, Ministry of Public Health, 2010

# *C. neoformans* classification

## Before 2002,

- 3 varieties of 5 serotypes
- *C. neoformans* var. *grubii* (serotype A),
- *C. neoformans* var. *gattii* (serotypes B and C),
- *C. neoformans* var. *neoformans* (serotype D),
- Hybrid serotype AD (Girish Kumar et al., 2006, Lin and Heitman, 2006).

## In 2002,

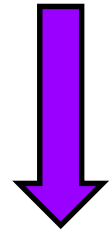
- *C. neoformans* var. *gattii* (serotypes B and C) was awarded species status and renamed *Cryptococcus gattii* (Lin and Heitman, 2006, Kwon-Chung et al., 2002).

# Method

## (Pigeon dropping collection)

- 50 samples were collected from Chon Buri province

*Columba livia*



Beach  
University area  
Village

Public park

Beach

# Result

*C. neoformans* in pigeon excreta in Chon Buri province

| location  |                    | N° samples |     |
|-----------|--------------------|------------|-----|
| district  | site               | total      | pos |
| Mueang    | beach              | 10         | 1   |
|           | university grounds | 10         | 1   |
|           | village            | 10         | 0   |
| Sri Racha | public park        | 10         | 1   |
| Pattaya   | beach              | 10         | 2   |
|           |                    | 50         | 5   |

10%

Serotype A

# Result

## Antifungal drug susceptibilities of *C. neoformans* isolates

| sample                         | MIC <sub>80</sub> * (µg/ml) |            |     |
|--------------------------------|-----------------------------|------------|-----|
|                                | AMB                         | ITZ        | FLZ |
| Env07                          | 0.25                        | 0.03       | 8   |
| Env18                          | 0.25                        | 0.06       | 8   |
| Env27                          | 0.25                        | 0.06       | 8   |
| Env32                          | 0.5                         | 0.125      | 16  |
| Env34                          | 0.125                       | 0.06       | 8   |
| clinical isolates <sup>1</sup> | 0.25-1                      | 0.03-0.125 | 1-4 |

\*MIC, minimal inhibitory concentration.

<sup>1</sup>unpublished data from strains isolated at Queen Savang Vadhana Memorial Hospital, Chon Buri.

No resistant strains

# Prevalence of different *C. neoformans* serotypes across South East Asia

10%

16%

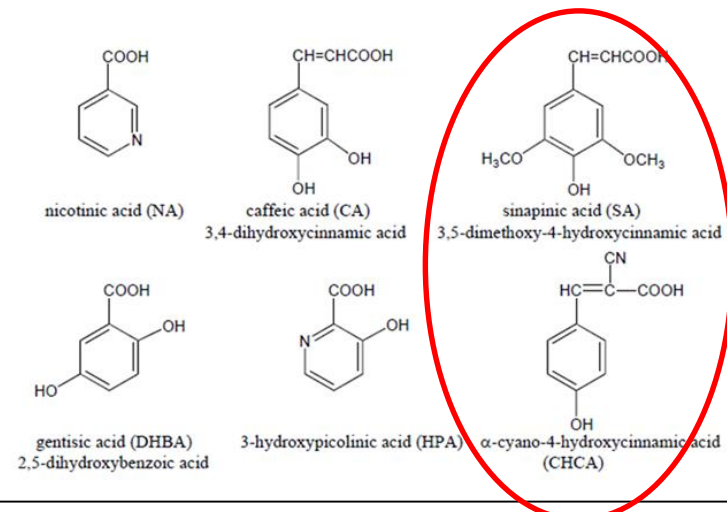
9%

|  | Country  | City        | Source                    | N° samples  |      | Serotype [%] |             |   |     | Reference               |
|--|----------|-------------|---------------------------|-------------|------|--------------|-------------|---|-----|-------------------------|
|  |          |             |                           | total       | pos. | A            | B/C         | D | AD  |                         |
|  | Thailand | Chon Buri   | pigeon excreta            | 50          | 5    | 100          |             |   |     | this study <sup>1</sup> |
|  |          | Chiang Mai  | pigeon excreta            | 55          | 9    | 100          |             |   |     | (Sriburee et al., 2004) |
|  |          | Bangkok     | pigeon excreta            | 11          | 1    |              | <i>n.d.</i> |   |     | (Soogarun et al., 2006) |
|  |          | Chiang Mai  | clinical                  | <i>n.a.</i> | 75   | 98.7         |             |   | 1.3 | (Sriburee et al., 2004) |
|  |          | countrywide | clinical                  | <i>n.a.</i> | 139  | 95.7         | 4.3         |   |     | (Poonwan et al., 1997)  |
|  | Vietnam  | Ho Chi Minh | HIV <sup>+</sup> patients | <i>n.a.</i> | 100  | 100          |             |   |     | (Day et al., 2011)      |
|  |          | Ho Chi Minh | HIV <sup>+</sup> patients | <i>n.a.</i> | 51   | 72.5         | 27.5        |   |     | (Day et al., 2011)      |
|  | Cambodia | Phnom Penh  | HIV <sup>+</sup> patients | <i>n.a.</i> | 402  | 98.5         | 1.5         |   |     | (Sar et al., 2004)      |
|  | Malaysia | countrywide | clinical                  | <i>n.a.</i> | 96   | 88.5         | 11.5        |   |     | (Tay et al., 2010)      |

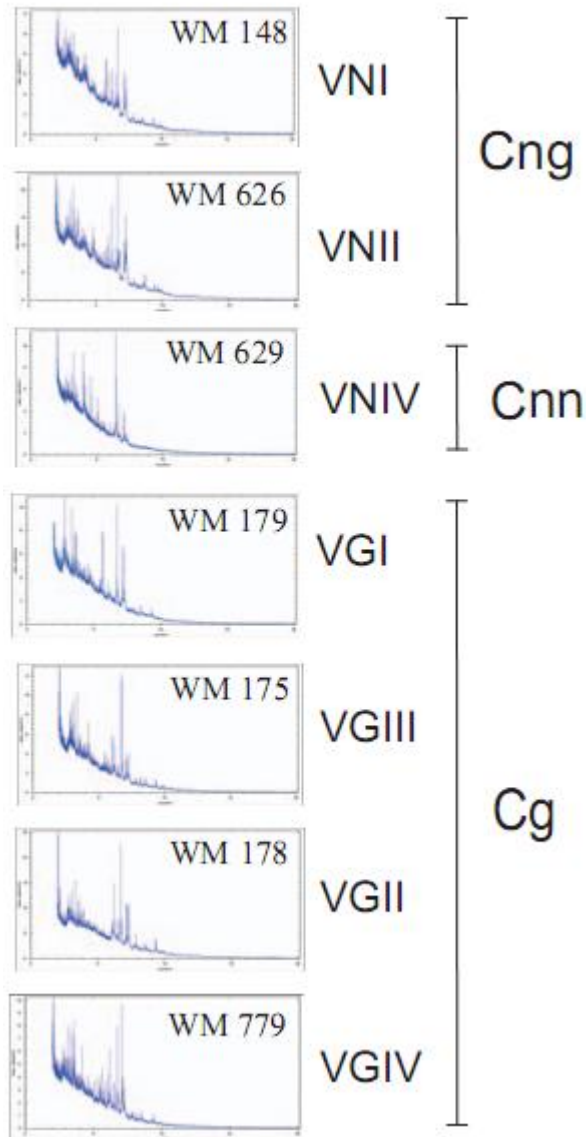
Taken from Tangwattanakuleporn M. et al, Med Mycol J, 2013

# Method

- Sample preparation (n=400 from CU) by using EtOH from colony  
(can not directly use a colony)
- Spot onto the plate
- Drop **the matrix** to cover the dried spot sample
  - 10 mg/ml of 4-hydroxy- $\alpha$ -cyano-cinnamic acid or 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid) in an aqueous solvent solution comprising 0.1% aqueous trifluoroacetic acid (TFA) and acetonitrile in a ratio of 70/30 (v/v)
- Laser shooting
- Interpretation by using mass spectra and confirmation with PCR-RFLP



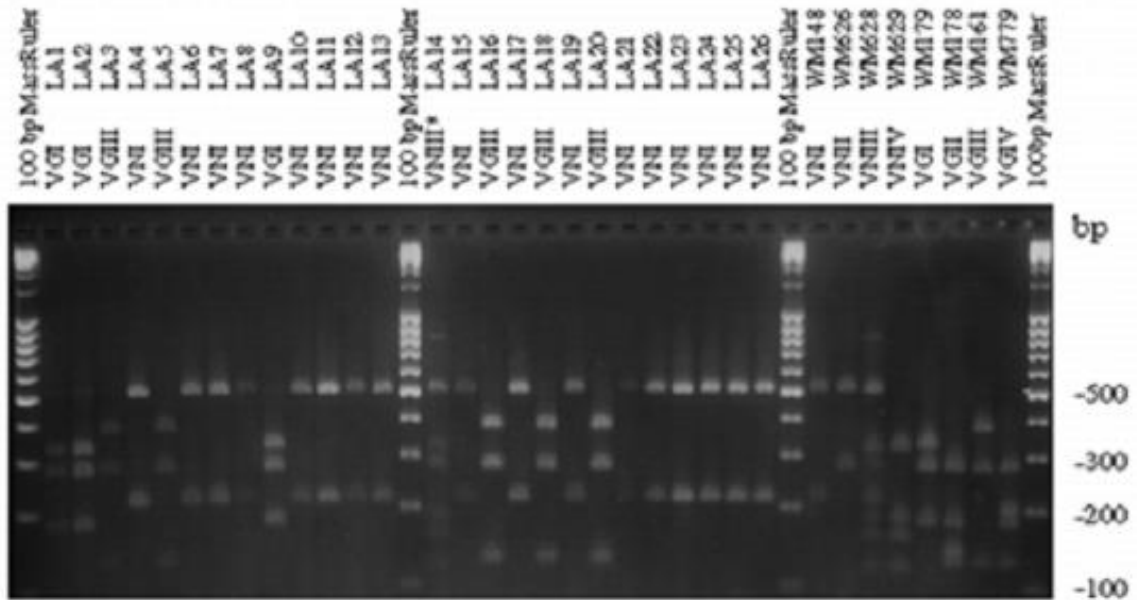
# MALDI-TOF



Firacative C, et. al., PLOS ONE, 2012

# PCR-RFLP

Amplification of URA5 gene and double digestion with Sau96I and HhaI



Meyer W, et al, Emerging Infectious Diseases, 2003

# THANK YOU FOR YOU ATTENTION



Institute for Medical Microbiology,  
University Medical Center Göttingen (UMG)



Asso. Prof. Dr. Ariya Chindamporn  
Navaporn worasilchai



# Opportunity for fungal research in Indonesia.

- Representative in each region
- Sample collection
- Sample preparation
- Sample sending



200 samples

MALDI-TOF







# Opportunity for fungal research in Indonesia

## Why fungal identification is importance?

**Table 1.** Spectrum of fungal isolates in all primarily sterile sites (left) and in blood cultures (right)

|                          |             |             |
|--------------------------|-------------|-------------|
| <i>C. albicans</i>       | 328 (58.5%) | 250 (58.4%) |
| <i>C. glabrata</i>       | 107 (19.1%) | 80 (18.7%)  |
| <i>C. parapsilosis</i>   | 45 (8.0%)   | 40 (9.3%)   |
| <i>C. tropicalis</i>     | 42 (7.5%)   | 27 (6.3%)   |
| <i>C. kefyr</i>          | 11 (2.0%)   | 9 (2.1%)    |
| <i>C. krusei</i>         | 8 (1.4%)    | 7 (1.6%)    |
| <i>C. guilliermondii</i> | 4           | 2           |
| <i>C. dubliniensis</i>   | 3           | 3           |
| <i>C. inconspicua</i>    | 3           | 3           |
| <i>C. catenulata</i>     | 1           | 1           |
| <i>C. colliculosa</i>    | 1           | 1           |
| <i>C. famata</i>         | 2           | 2           |
| <i>C. intermedia</i>     | 1           | 1           |
| <i>C. lusitaniae</i>     | 1           |             |
| <i>C. norvegensis</i>    | 2           |             |
| <i>C. rugosa</i>         | 1           | 1           |
| <i>C. utilis</i>         | 1           | 1           |
| Total                    | n = 561     | n = 428     |

### Prevalence in Germany

(Borg-von Zepelin, 2007)

**Table 1.** In Vitro Activity of Currently Used Antifungal Agents

| Fungal species                            | FLU | ITRA | POSA | VOR | AMB | Echinocandins <sup>a</sup> |
|-------------------------------------------|-----|------|------|-----|-----|----------------------------|
| <i>Aspergillus fumigatus</i>              | –   | +    | +    | +   | +   | +                          |
| <i>Aspergillus flavus</i>                 | –   | +    | +    | +   | +   | +                          |
| <i>Aspergillus terreus</i>                | –   | +/-  | +    | +   | +/- | +                          |
| <i>Candida albicans</i>                   | +   | +    | +    | +   | +   | +                          |
| <i>Candida krusei</i>                     | –   | +/-  | +    | +   | +/- | +                          |
| <i>Candida glabrata</i>                   | +/- | +/-  | +/-  | +/- | +   | +                          |
| Other <i>Candida</i> species <sup>b</sup> | +   | +    | +    | +   | +   | +/-                        |
| <i>Cryptococcus neoformans</i>            | +   | +    | +    | +   | +   | –                          |
| <i>Coccidioides</i> species               | +   | +    | +    | +   | +   | –                          |
| <i>Blastomyces dermatitidis</i>           | +/- | +    | +    | +   | +   | –                          |
| <i>Histoplasma capsulatum</i>             | +   | +    | +    | +   | +   | –                          |
| <i>Fusarium</i> species                   | –   | –    | +/-  | +/- | +/- | –                          |
| Zygomycetes                               | –   | +/-  | +    | –   | +   | –                          |
| <i>Scedosporium apiospermum</i>           | –   | –    | +    | +   | +/- | –                          |
| <i>Scedosporium prolificans</i>           | –   | –    | –    | –   | –   | –                          |
| <i>Trichosporon</i>                       | –   | –    | ND   | +   | +/- | –                          |

**NOTE.** +, in vitro activity; –, no in vitro activity; +/- modest in vitro activity; AMB, amphotericin B; FLU, fluconazole; ITRA, itraconazole; ND, no data; POSA, posaconazole; VOR, voriconazole.

<sup>a</sup> Caspofungin, micafungin, and anidulafungin.

<sup>b</sup> *Candida parapsilosis* is less susceptible in vitro to the echinocandins.

Leventakos, 2010

*C. glabrata* is the second most frequent cause of Candidiasis and highly resistant to azole antifungal drugs

# *C. glabrata* resist to Fluconazole and Voriconazole

## Why fungal identification is importance?

TABLE 6. Geographic variation in the in vitro susceptibilities of *C. albicans*, *C. glabrata*, and *C. tropicalis* to fluconazole and voriconazole<sup>a</sup>

| Region             | Antifungal agent | <i>C. albicans</i>     |     | <i>C. glabrata</i>     |      | <i>C. tropicalis</i>   |     |
|--------------------|------------------|------------------------|-----|------------------------|------|------------------------|-----|
|                    |                  | No. of isolates tested | % R | No. of isolates tested | % R  | No. of isolates tested | % R |
| Asia-Pacific       | Fluconazole      | 18,125                 | 0.9 | 3,368                  | 13.5 | 3,120                  | 8.1 |
|                    | Voriconazole     | 17,298                 | 0.9 | 3,259                  | 7.7  | 2,989                  | 4.7 |
| Europe             | Fluconazole      | 52,127                 | 1.2 | 8,642                  | 16.0 | 3,930                  | 2.6 |
|                    | Voriconazole     | 50,926                 | 1.1 | 8,492                  | 9.9  | 3,817                  | 3.5 |
| Africa/Middle East | Fluconazole      | 4,566                  | 0.6 | 564                    | 19.1 | 350                    | 2.9 |
|                    | Voriconazole     | 4,529                  | 0.3 | 541                    | 9.1  | 348                    | 2.6 |
| Latin America      | Fluconazole      | 10,288                 | 2.4 | 1,472                  | 14.0 | 2,525                  | 3.0 |
|                    | Voriconazole     | 9,830                  | 1.9 | 1,434                  | 9.6  | 2,423                  | 4.4 |
| North America      | Fluconazole      | 4,644                  | 5.1 | 2,106                  | 20.5 | 740                    | 3.6 |
|                    | Voriconazole     | 4,608                  | 3.7 | 2,098                  | 15.3 | 729                    | 4.7 |

<sup>a</sup> Isolates were obtained from 124 institutions. Fluconazole and voriconazole disk diffusion testing was performed in accordance with CLSI document M44-A (16). The interpretive breakpoints (zone diameters) for resistance were as follows: fluconazole,  $\leq 14$  mm; voriconazole  $\leq 13$  mm.

| Species                        | Molecular type | Scores*     |
|--------------------------------|----------------|-------------|
| <i>Cryptococcus neoformans</i> | VNI            | 2.055–2.445 |
|                                | VNII           | 2.319–2.358 |
|                                | VNIII          | 2.135–2.161 |
|                                | VNIV           | 2.083–2.204 |
| <i>Cryptococcus gattii</i>     | VGI            | 2.012–2.711 |
|                                | VGII           | 2.055–2.166 |
|                                | VGIII          | 2.055–2.500 |
|                                | VGIV           | 1.882–1.945 |

\*2.300–3.000: highly probable species identification; 2.000–2.299: secure genus identification, probable species identification; 1.700–1.999: probable genus identification.